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(54) Title: CYTOKININ OXIDASE

(57) Abstract

An isolated protein which exhibits cytokinin oxydizing activity selected from the group consisting of SEQ. ID No. 1, a protein having an amino acid sequence which includes the amino acid sequence of SEQ. ID No. 1, a protein having an amino acid sequence which includes a portion of the amino acid sequence of SEQ. ID No. 1, the included portion being at least about 20 amino acid residues in length and conferring the cytokinin oxidizing activity on the protein, and a protein including an amino acid sequence with at least about 65 % sequence identity to SEQ. ID No. 1, the remainder of amino acid residues being conservatively substituted. Nucleic acids encoding proteins which exhibit cytokinin oxidizing activity and related products and methods are also disclosed.

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CYTOKININ OXIDASE

BACKGROUND OF THE INVENTION

The present invention relates to a purified plant cytokinin oxidizing enzyme (*ckx1*) from *Zea mays*, the complete amino acid sequence of which has been elucidated, and to isolated nucleotide sequences encoding the enzyme. The invention further relates to novel methods for moderating the concentration of the enzyme and similar enzymes in plants in order to affect plant cell growth and death. Applications of the invention include the regulation of the production of *ckx1* in plant roots to affect pathogenesis, the regulation to alter plant habit, and the bulk production of *ckx1* enzyme for use in a plant biochemical assay.

Plant cytokinins are a class of plant hormones which, when combined with auxin, control cell division, promote shoot development from callus, release lateral buds from dormancy, and regulate plant structure and growth in a variety of ways. The naturally occurring active cytokinins in most higher plants are free-base zeatin (6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine) (hereinafter Z), and its 9-riboside (hereinafter ZR). Plant tissues normally contain, therefore, Z, ZR, and smaller amounts of N^6-(Δ^2-isopentenyl)adenine (hereinafter, iP) derived from biosynthetic precursors. Elevated cytokinin levels are associated with the development of seeds in higher plants, and have been demonstrated to coincide with maximal mitotic activity in the endosperm of developing maize kernels and other cereal grains. Exogenous cytokinin application (via stem injection) has been shown to directly correlate with increased kernel yield in maize. In addition, plant cells transformed with the *ipt* gene from *Agrobacterium tumefaciens* (encoding a

dimethylallylpyrophosphate:5'-AMP transferase capable of increasing cellular production of Z and ZR) showed increased growth corresponding to an increase in endogenous cytokinin levels upon induction of the enzyme. Thus, given the biosignificance of cytokinins to the growth of plants, the ability to manipulate cytokinin levels in higher plant cells is of great commercial and scientific interest.

The action of cytokinin oxidase is a major method of effective cytokinin catabolism in plant cells. This inactivation of cytokinin is accomplished by the oxidative removal of the side chain from cytokinin free bases (or their ribosides) in the presence of molecular oxygen. An example of this reaction with iP is shown in Figure 1a. Although the exact chemical mechanism for this reaction is unknown, it is suspected that the enzyme is reduced during the deprotonation of iP to N^6-(Δ^2-isopentenylimino)purine. The purine is then hydrolyzed into adenine and intermediate 3-methyl-2-butenal (Figure 1b).

While the electron acceptor responsible for reoxidizing the reduced enzyme in plant cells is not known, molecular oxygen can do so *in vitro*. Alternatively, the reduced enzyme may be reoxidized *in vitro* by intermediates such as Cu^{+2}/imidazole complexes or the artificial electron acceptor dichlorophenolindophenol (DCPIP).

Cytokinin oxidases are known to remove cytokinins from plant cells after cell division, and have also been postulated to be involved in the senescence process. Cytokinin oxidase activities have been shown to positively correlate to the mitosis of endosperm cells in maize kernels, along with the increase in natural cytokinin concentrations. Oxidase activity increases shortly after the increase in endogenous cytokinin levels. A similar correlation was demonstrated with artificially increased cytokinin levels in transgenic

tobacco. Thus, expression of cytokinin oxidases is thought to be involved in the maintenance of hormonal homeostasis in developing plant cells. Because cytokinin oxidases appear to be substrate-inducible, they act in a negative regulatory fashion to reduce elevated cytokinin levels back to basal values. This substrate induction of cytokinin oxidase activity is a significant barrier to potential commercial applications which attempt to manipulate cytokinin levels in transgenic plants through increased cytokinin production.

Cytokinin oxidases have been discussed for a number of plant species, including *Vinca rosea*, beans (*Phaseolus vulgaris* and *lunatus*), wheat (*Triticum aestivum*), tobacco (*Nicotiana tabacum*), *Dianthus caryophyllus*, soy (*Glycine max*), and maize (*Zea mays*). All of these plant cytokinin oxidases have a similar substrate preference for iP and Z, but show limited or no reactivity with bulky, reduced, or aromatic side chain cytokinins. All also exhibit enhanced activity in the presence of copper plus imidazole. However, these enzymes show substantial variation in both specific activity and molecular weight. This is thought to be linked to the occurrence of glycosylated and unglycosylated variants of the protein, both between and within species.

In the case of the glycosylated cytokinin oxidase, the heavily glycosylated protein may present a carbohydrate-rich surface, preventing antibody formation against peptide epitopes. The glyco-epitopes to which antibodies are raised under these conditions are non-specific, and may prevent isolation of the protein, or clones containing the gene which encodes it, via immuno-chromatography or other immunology-based means. An earlier reported attempt to isolate the gene for maize

cytokinin oxidase (*ckx1*) by immunoscreening of maize cDNA library expression products (Burch, 1992) was unsuccessful.

As demonstrated, the full amino acid sequence and encoding DNA for a cytokinin oxidase has been a long sought after goal in modern plant physiology.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a means by which recombinant cytokinin oxidase may be produced in quantity so that the effects of cytokinin oxidase on plant growth and metabolism may be studied. It is also an object of the present invention to provide a means for the modification of cytokinin oxidase production in plant cells, *in vivo*, in order to modulate the endogenous cytokinin level of plant cells to effect altered pathogen resistance and plant growth properties.

The present invention, therefore, is directed to a novel, isolated and substantially purified plant cytokinin oxidizing enzyme, (*ckx1*), having a molecular weight most preferably of about 60 kD, a sequence length of from about 505 to 565 amino acid residues, preferably 525 to 545 amino acid residues, and most preferably 534 amino acid residues, and having cytokinin inactivating activity. The present invention is also directed to a protein having an amino acid sequence which includes the amino acid sequence of *ckx1* (SEQ. ID NO. 1). The invention is directed as well to a protein which has cytokinin inactivating activity and which includes a portion of the amino acid sequence of *ckx1* at least about 20 amino acid residues in length, where the included portion of the *ckx1* sequence confers the cytokinin inactivating activity on the protein. The invention is directed to proteins which have cytokinin inactivating activity and have at least about 65% sequence identity to

ckx1 and most preferably at least about 95% sequence identity to *ckx1*, with the remaining amino acids being conservatively substituted.

The invention is directed, moreover, to substantially isolated nucleic acid polymers encoding *ckx1* or a cytokinin oxidizing homolog thereof. The nucleic acid polymer most preferably has a nucleic acid sequence of SEQ. ID NO. 3 or the predictable variants thereof described in SEQ. ID NO. 10. The invention is also directed to a substantially isolated nucleic acid polymer which contains a portion of SEQ. ID NO. 2, SEQ. ID NO. 3, or a nucleic acid polymer described by SEQ. ID NO. 10, the portion being at least 60 bp in length. In addition, the invention is directed to nucleic acid polymers which are able to hybridize with SEQ. ID NO. 2, SEQ. ID NO. 3, or a nucleic acid polymer described by SEQ ID NO. 10, under conditions of 0.5X to 2X SSC buffer, 0.1% SDS, and a temperature of 55-65°C. Nucleic acid polymers which encode cytokinin oxidases and meet the above requirements encode proteins of sufficient similarity to *ckx1* to be generally recognized as equivalents of *ckx1* among those skilled in the biochemical arts.

The invention is also directed to a host cell incorporating a vector containing the aforementioned DNA, and to a method for producing *ckx1* or a homolog thereof using such a host cell. The method preferably comprises first ligating DNA encoding the aforementioned *ckx1* or a segment or homolog thereof and an appropriate promoter (such as the *RB7* root-specific promoter, Conkling, M.A., et al., U.S. Pat. No. 5,459,252; or *CaMV35S* promotor, Odell, 1985), or a combination of promoters (Hoffman, Patent No. 5,106,739) 3) into an appropriate DNA vector (for instance, pBIN19 for use in *Agrobacterium tumefaciens*). The vector construct may then be directly

transformed into a host cell, such as *Pichia pastoris* (described in Example 2). It may also be incorporated into a secondary vector for transformation into a host cell, such as *Agrobacterium tumefaciens*, and transformed into a plant cell host (described in Example 4 with *Nicotiana tabacum*).

Alternatively, for production of larger amounts of the enzyme, the DNA encoding *ckx1*, or a portion thereof, may be transformed into *Pichia*, according to the methods described in Example 2, or in Su, et al., 1996 and Skory, et al., 1996. When transformed into *Pichia spp.*, *ckx1* is secreted into the culture medium because of the presence of a secretory signal peptide at the N-terminus of the *ckx1* coding region. Thus, active enzyme may be readily purified from bulk *Pichia* cultures without a lysing step. *ckx1* produced in such a manner may be used in a biochemical assay to determine unknown concentrations of cytokinin in biological samples, according to the method of Example 3.

A plant host cell generated by the above method may be regenerated into an entire plant. Depending on the promoter used in the vector construct, *ckx1* may be produced constitutively or by induction through natural or artificial environment factors. Plants transformed with vectors containing tissue-specific or trauma-specific promoters and a sequence encoding *ckx1* can exhibit altered resistance to certain cytokinin-linked plant pathologies, such as infection by certain nematodal or fungal species.

The discoveries described herein provide an important analytical tool for, and a critical link in, the development of methods by which the manipulation of cytokinin oxidase activity may be used to either inhibit or enhance a variety of cell growth functions in plants in a desired manner. Possible uses include the

development of commercial plants with increased grain production, disease resistance, or with more desirable secondary growth characteristics. The enzyme and its encoding nucleic acids have important uses in the study of plant cell growth cycles and senescence. In addition, many other pharmaceutical and agricultural uses for *ckx1* and its gene may be discovered. The enzyme, methods of expressing the enzyme, and methods for its use are described in greater detail below.

Other features and objects of the present invention will be in part apparent to those skilled in the art and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE FIGURES, SEQUENCE IDENTIFICATIONS, AND DEFINITIONS

The invention is further disclosed and illustrated by the accompanying figures.

FIG. 1a & b show an exemplary reaction catalyzed by cytokinin oxidase (Brownlee, 1975), and its putative mechanism as described above, based on Hare, 1994.

FIG. 2 shows agarose gel electrophoresis of RT-PCR DNA fragments which demonstrate that the introns of the *ckx1* gene have been correctly identified.

FIG. 3 shows the standard spectrophotometric absorbance curve (590 nm) obtained when using *ckx1* to assay cytokinin concentrations in solution.

FIG. 4 is a diagram of DNA plasmid pJL7.

FIG. 5 is a diagram of DNA plasmid pBI121.

FIG. 6 is a diagram of DNA plasmid pROM8.

FIG. 7 is a diagram of DNA plasmid pROM9.

FIG. 8 is a diagram of DNA plasmid pROM22.

FIG. 9 is a diagram of DNA plasmid pROM24.

FIG. 10 is a diagram of DNA plasmid pROM26.

FIG. 11 is a diagram of DNA plasmid pROM28.

FIG. 12 is a diagram of DNA plasmid pROM29.

FIG. 13 is a diagram of DNA plasmid pROM30.

FIG. 14 is a diagram of DNA plasmid pROM32.

FIG. 15 is a diagram of DNA plasmid pROM43.

All Sequence Identification abbreviations of amino acids and nucleotides conform to USPTO and WIPO standards.

SEQ. ID NO. 1 lists the amino acid sequence of naturally occurring *ckx1* derived from *Zea mays*. This sequence was predicted from the genomic DNA derived nucleotide sequence encoding *ckx1*.

SEQ. ID NO. 2 lists the genomic DNA sequence encoding *ckx1*, including introns.

SEQ. ID NO. 3 lists the coding DNA sequence for *ckx1*, with introns excluded. This sequence has been reconstructed in pROM22 of Example 2.

SEQ. ID NO. 4 lists the amino acid sequence of an internal tryptic digest fragment of *ckx1*.

SEQ. ID NO. 5 lists the amino acid sequence of an internal tryptic digest fragment of *ckx1*.

SEQ. ID NO. 6 lists the nucleic acid sequence of a degenerate DNA probe used to isolate the genomic DNA encoding *ckx1*, as described in Example 1. Note that the residues designated "n" in the sequence are the artificial base inosine (I). Normal conventions have been followed for the indication of degeneracies in the sequence.

SEQ. ID NO. 7 lists the nucleic acid sequence of a degenerate DNA probe used to isolate the genomic DNA encoding *ckx1*, as described in Example 1. Note that the residues designated "n" in the sequence are the artificial base inosine (I). Normal conventions have been followed for the indication of degeneracies in the sequence.

SEQ. ID NO. 8 lists the nucleic acid sequence of a degenerate DNA probe used to isolate the genomic DNA

encoding *ckx1*, as described in Example 1. Note that the residues designated "n" in the sequence are the

artificial base inosine (I). Normal conventions have been followed for the indication of degeneracies in the sequence.

SEQ. ID NO. 9 lists the nucleic acid sequence of a degenerate DNA probe used to isolate the genomic DNA encoding *ckx1*, as described in Example 1. Note that the residues designated "n" in the sequence are the artificial base inosine (I). Normal conventions have been followed for the indication of degeneracies in the sequence.

SEQ. ID NO. 10 lists the degenerate DNA sequence encoding *ckx1*. As is well known in the art, the several DNA molecules indicated by this group encode a protein with the amino acid sequence of SEQ. ID NO. 1, also known as *ckx1*. This group follows the conventional rules of degeneracy in the genetic code. Special modifications necessary for expression in certain organisms which do not follow these conventions could easily be made by an individual of ordinary skill in the art.

SEQ. ID NO. 11 lists the sequence of a synthetic primer used in the PCR removal of introns in example 2.

SEQ. ID NO. 12 lists the sequence of a synthetic primer used in the PCR removal of introns in example 2.

SEQ. ID NO. 13 lists the sequence of a synthetic primer used in the PCR removal of introns in example 2.

SEQ. ID NO. 14 lists the sequence of a synthetic primer used in the PCR removal of introns in example 2.

SEQ. ID NO. 15 lists the sequence of a synthetic primer used in the PCR removal of introns in example 2.

SEQ. ID NO. 16 lists the sequence of a synthetic primer used in the PCR removal of introns in example 2.

SEQ. ID NO. 17 lists the sequence of a synthetic linker construct used in example 2.

SEQ. ID NO. 18 lists the sequence of a synthetic linker construct used in example 2.

SEQ. ID NO. 19 lists the sequence of a synthetic primer used in PCR to obtain the tobacco RB7 promoter in example 4.

SEQ. ID NO. 20 lists the sequence of a synthetic primer used in PCR to obtain the tobacco RB7 promoter in example 4.

As used herein, a "substantially purified protein" means that the protein is separated from a majority of host cell proteins normally associated with it or that the protein is synthesized in substantially purified form, such synthesis including expression of the protein in a host cell from a nucleic acid polymer exogenously introduced into the cell by any suitable gene-therapy delivery means.

A "substantially isolated nucleic acid polymer" means that the mixture which comprises the nucleic acid polymer of interest is essentially free of a majority of other nucleic acid polymers normally associated with it. A "nucleic acid polymer" includes a polymer of nucleotides or nucleotide derivatives or analogs, including for example deoxyribonucleotides, ribonucleotides, etc. Genomic DNA, cDNA and mRNA are exemplary nucleic acid polymers.

The terms "regulate transcription," "modify transcription," "regulate production," and "modify production," is intended to include promotion and/or repression of transcription or mRNA or production/translation of a protein.

The term "expression regulatory sequence" means a nucleic acid polymer sequence ligated to a protein encoding sequence which, when introduced into a host cell, either induces or prevents expression of that protein. These sequences may or may not also encode

proteins used in their regulatory mechanism. Examples of expression regulatory sequences include the *CaMV* promoter, the *ocs* terminator, and the *tet* operator sequences.

The term "gene" is intended to include both endogenous and heterologous genes, and specifically, both genomic DNA which encodes a target protein in a naturally occurring cell, and also cDNA encoding the target protein, for example, wherein the cDNA is a part of a nucleic acid construct such as a plasmid vector or virus which has been introduced into a cell or a cDNA produced by RT-PCR.

The term "vector" is intended to include any physical or biochemical vehicle containing nucleic acid polymers of interest, by which those nucleic acid polymers are transferred into a host cell, thereby transforming that cell with the introduced nucleic acid polymers. Examples of vectors include DNA plasmids, viruses, particle gun pellets, and bacteria such as *Agrobacterium tumefaciens*. The term "primary vector" is intended to mean the first vector used in a transformation series, either as one step (*e.g.* a plasmid used to transform a yeast cell), or with a "secondary vector" (*e.g.* a plasmid used to transform *Agrobacterium tumefaciens*, which is later used to transform a plant cell).

The term "host cell" is intended to mean the target cell for vector transformation, in which the transferred nucleic acid polymer will be replicated and/or expressed.

The term "conservative substitution," in the context of amino acid sequences, means the substitution of one amino acid in the sequence with another with a side chain of similar size and charge. An example of a conservative substitution would be substituting glutamine for asparagine. Conservative substitutions in a protein

sequence which would be expected to have minimal to no impact on protein structure or function can be readily devised by a person of ordinary skill in the biochemical arts.

The term "plant products" means any cellular material produced by a plant, especially those which may be used for propagation of the plant. Plant products include seeds, rhizomes, leaves, meristem, roots, and buds.

The term "SSC buffer" means a solution of 8.765 g NaCl and 4.41 g sodium citrate in 1 liter of water, pH adjusted to 7.0 by titrimetric addition of 10 N NaOH solution.

The contents of each of the references cited herein are being incorporated by reference in their entirety.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to newly sequenced cytokinin oxidase isolated from the maize plant, *Zea mays*, designated *ckx1*, which exhibits a substrate specific cytokinin oxidizing activity. The enzyme has been linked to the development and maturation of kernels in maize, as well as the senescence of plant tissues. The control of these plant functions appears to be achieved by the degradation of endogenous and exogenous cytokinin concentrations. Because *ckx1* efficiently oxidizes unsaturated-side-chain free cytokinins and their ribosides, such as iP, Z, and ZR (which also induce production of the enzyme when applied exogenously or endogenously), it is thought that *ckx1* rigorously controls the availability of these active cytokinins as a part of plant growth regulation.

During the course of developing the claimed invention, the applicant encountered several difficulties which may explain why the search for a cytokinin oxidase gene has been heretofore unsuccessful. The protein is

expressed at very low levels, and only at certain periods of the plant growth cycle. Therefore, screening a λ-cDNA maize library in E. coli may not yield *ckx1* simply because the gene was not expressed, or not expressed in detectable amounts, when the library was taken. Also, isolation of the protein may have been a problem for some researchers, since its active form does not survive gel filtration. Only through the development of his own screening process, described in Example 1, was the applicant able to isolate the protein in sufficient purity for tryptic digestion and subsequent sequencing.

Even after the protein was purified, obtaining the gene sequence for *ckx1* proved to be difficult. Apparently, the N-terminal end of *ckx1* is blocked, so a straightforward Edman degradation determination of its N-terminal sequence was impossible. Only small, internal sequences were determinable after tryptic digestion of the protein, SEQ. ID NO. 4 & 5. Because the location of these fragments in the protein's amino acid sequence was unknown, several problems had to be overcome in order to successfully probe the *Zea mays* genome for the *ckx1* gene, as detailed in Example 1. The small size of the sequenced peptides necessitated using a "bookended" criteria (one probe at either end of the replicated DNA) in order to eliminate non-*ckx1* DNA from either side of the *ckx1* gene. One could be reasonably certain that the DNA between two probes would be part of the *ckx1* gene. A hybridization/amplification product size criteria of 300 bp was also necessary in order to distinguish between dimerized degenerate primers and PCR products. A simple degenerate strategy was unlikely to work because of the high degeneracy inherent in the particular amino acid sequences available from the tryptic peptides. Initially, when a standard degenerate nucleotide strategy was used, utilizing all possible oligonucleotides encoding the interior amino acid sequences, the low concentration of

specifically binding primer was not sufficient for the initiation of PCR amplification. The applicant was able to overcome this problem only by using inosine containing degenerate probes with a broader specificity. Also, several of the degenerate probes were too close together to yield products of the target size, or not in the right order in the gene to produce "bookended" products. Only probes SEQ ID NO. 6, 7, 8, & 9 proved to be of use in isolating the *ckx1* gene.

The identity of the isolated gene was verified by two independent methods. First, it was verified by testing the affinity of goat antisera to an *Escherichia coli* produced translation of SEQ. ID NO. 3 for *ckx1* in maize kernel extracts, as per Example 1. Second, it was confirmed by expressing SEQ. ID No. 3 in *Pichia pastoris* resulting in the secretion of active cytokinin oxidase as per Example 2.

SEQ. ID NO. 2 lists the complete genomic DNA sequence of *ckx1*, which yields the glycosylated form of the protein when expressed in *Zea mays*. The predicted location of the introns in the genomic sequence was verified using the reverse transcriptase- polymerase chain reaction to find the actual transcribed length of RNA, as per Example 1. SEQ. ID NO. 3 lists the coding DNA sequence for *ckx1*, which yields the amino acid sequence set forth in SEQ. ID NO. 1.

The state of the art of molecular biology is now sufficiently advanced that minor alterations can be made to a DNA sequence with relative ease and precision. A moderately skilled laboratory technician can follow the directions of one of the commercially available site-directed mutagenesis kits (for instance, the GeneEditor™ offered by Promega Corp., Madison, Wisconsin) to effect any number of changes to a DNA nucleotide sequence. Also well known are the general rules governing the genetic code, by which triplet nucleotide codons are translated

into an amino acid sequence by standard biochemical processes. Thus, the applicant considers the group of DNA sequences denoted by the consensus sequence of SEQ. ID NO. 10, which code for the amino acid sequence of *ckx1* in SEQ. ID. NO. 1, to be within the present invention. Although some variation in the genetic code and the GC% content occurs amongst some phyla, the rules governing these variations have also been well documented, and are within the reasonable skill of one versed in the molecular genetic arts. Thus, the applicant also considers any other nucleic acid sequence which encodes the amino acid sequence SEQ. ID NO. 1 to be within the scope of the present invention.

In addition, although the understanding of the field of protein biochemistry is not as complete as that of molecular genetics, the person or ordinary skill in the art of biochemistry is capable of predicting, with reasonable certainty, when certain substitutions to the primary amino acid sequence structure of a protein will not result in any appreciable modification of a protein's structure or function. Such conservative substitutions are made by replacing an amino acid in the sequence with another containing a side chain with like charge, size, and other characteristics. For instance, the amino acid alanine, which has a small nonpolar methyl side chain, generally can be replaced by glycine, an amino acid which has a small nonpolar hydrogen side chain, without any noticeable effects. Likewise, the amino acid asparagine, with a moderately bulky, polar ethamide side chain, usually can be replaced with glutamine, which has a moderately bulky, polar propamide side chain, without noticeable effects. To the extent that such conservative substitutions can be made while retaining 65%, preferably 80% or more identity to SEQ. ID NO. 1 and cytokinin oxidizing activity, such altered proteins are within the scope of the present invention.

Cytokinin oxidases are known to exist in a variety of non-glycosylated and glycosylated forms in several species of higher plants, including maize. This modification is thought to be involved in compartmentalizing cytokinin oxidases for various uses inside and outside the cell. The extent of glycosylation of the enzyme may also account for the wide variety of molecular weights observed between the cytokinin oxidases of various species. However, because substrate specificity, a requirement of molecular oxygen for activity, and copper concentration reaction rate effects *in vitro* are highly conserved among all higher plant cytokinin oxidases, a common domain and active site structure is believed responsible for the cytokinin oxidizing activity of all enzymes. Thus, the present invention is also directed to a protein which exhibits cytokinin oxidizing activity and which contains an amino acid sequence at least about 20 amino acids in length which is 90% identical (or would be identical with conservative amino acid substitutions) to a similarly sized portion of SEQ. ID NO. 1.

Another object of the current invention is the regulated production of *ckx1* in various host cells, either for later bulk isolation, or regulated intracellular production. For instance, *ckx1* may be produced in unglycosylated form in prokaryotes, such as *E. coli*, as illustrated in example 1. More preferably, the protein may be produced in eukaryotes, as illustrated by the *Pichia* production of example 2. An added benefit of producing the protein in *Pichia* is that the protein is secreted into the culture media where it may be readily purified. Alternatively, the protein may be produced in higher eukaryotes, more preferably plants, such as the *Nicotiana* constructs of example 4 either as the glycosylated or non-glycosylated forms. Other examples of suitable host plant cells include Zea *mays*,

Arabidopsis thaliana, *Brassica spp*, and *Oryza sativa*. As illustrated, *ckx1* can be produced in plants either in an unregulated fashion, as shown here under the *CaMV* promoter, regulated by an artificial stimuli, as shown here under a *tet* operator combined with a *CaMV* promoter, and regulated by an environmental stimuli, as shown here under the *RB7* promotor, which induces root-specific production of a protein in response to nematodal attack.

The several aspects of the present invention, including the *ckx1* protein and the nucleic acid polymers which encode it, the cytokinin oxidizing activity of enzymes such as the *ckx1* enzyme, and the regulation of cytokinin levels in plant cells by *ckx1*, collectively enable several practical applications, including both agricultural and research uses.

The applicant has devised an application for bulk cytokinin oxidase which greatly facilitates plant physiology research. Cytokinin oxidase can be reoxidized after oxidizing cytokinins by the synthetic oxidizing agent dichlorophenolindophenol (DCPIP). DCPIP demonstrates a reduced absorbance at 590 nm upon reduction, which can be spectrophotometrically quantified. The cytokinin concentration in a sample may be determined indirectly by measuring the decrease in oxidized DCPIP as *ckx1* oxidizes the cytokinins present in the sample. More sensitive redox dyes are also available to increase the sensitivity of the assay. Therefore, it is another object of the present invention to provide a simple, fast, and effective means to assay cytokinin concentrations in a sample.

The applicant has also devised an application for the modified production of *ckx1* in plants. Cytokinins are associated with several types of plant pathogenesis, including the formation of nematode feeder cells, and

fungal invasion of plant tissues. Thus, the pathogen-exposure-regulated production of *ckx1* can modify the efficacy of pathogenesis through cytokinin-utilizing mechanisms such as that utilized by the root-knot nematode, *Meloidogyne spp*. (Bird, et al., 1980), or fungal species such as *Ustilago maydis* . Thus, it is an object of the present invention to provide a method of moderating cytokinin-mediated pathogenesis by transforming a plant with a *ckx1* producing DNA construct.

The following examples illustrate the principles and advantages of the invention.

EXAMPLES

Example 1: Isolation of *ckx1* and characterization of its encoding sequence

Maize kernels were chosen as the raw material for purification because of the relatively high concentrations of cytokinin oxidase present about a week after pollination. Using the procedure set forth below, approximately 1.66 μg protein per kg maize yield may be obtained. Field grown maize (Pioneer 3180 or 3379) was hand pollinated, and immature ears were harvested between 5 and 8 days later (Dietrich, 1995). Kernels were harvested immediately, and frozen at -80°C until extraction. After being powdered in liquid nitrogen, the kernels were blended in 1 kg lots with 1700 ml of Buffer A(50 mM Tris, 5 mM EDTA, ascorbic acid 0.4% w/v; 10 mM β-mercaptoethanol, pH 8.5). Acid washed PVPP (200 g wet weight, equilibrated w/ Buffer A) was stirred in immediately. The slurry was filtered through Miracloth and centrifuged at 23,500 x g for 15 minutes to remove debris. Polyethyleneimine solution (5% v/v, pH 8.5) was added dropwise to the centrifuged supernatant, to a final concentration of 0.05%. After recentrifugation at 23,500

x g for 10 minutes, the supernatant was filtered through a 600 g pad of PVPP (prepared as above). Ammonium sulfate fractionation was performed, and the protein precipitating between 40% and 65% saturation was collected by centrifugation and dissolved in a minimum volume of Buffer B (10 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.5). Insoluble material was removed by centrifugation at 35,000 x g for 20 minutes. At this point, glycerol may be added to the supernatant to 10% v/v, allowing the protein to be stored at -80°C indefinitely without loss of activity.

After dialyzing the supernatant from the ammonium sulfate fractionation against Buffer B, the fraction was applied to a DEAE-cellulose column (Whatman DE-52, 500 ml, available from the Whatman Group, Clifton, New Jersey) at the rate of 10 ml/minute. After washing w/ buffer B, the column was eluted with a linearly increasing concentration of KCl in Buffer B, to 200 mM over 600 ml. Protein content was measured using the Bradford dye binding assay (Bradford, 1976). Fractions were analyzed for oxidase activity as described below.

Two assays were used to screen purification steps for cytokinin oxidase activity. The first was the Schiff base formation assay measuring the production of dimethylallylaldehyde from iP, as described by Liberos-Minotta (1995), which was used up through the DEAE column step. A second assay, developed by the applicant, was used in the remaining purification steps. In this assay, the transfer of reducing equivalents from isopentenyladenine to dichlorophenolindophenol (DCPIP), catalyzed by *ckx1*, allows the observance of reactivity by measuring absorbance change at 590 nm. In a final volume of 250 µl, the assay contains: 100 mM Phosphate buffer, pH 7.0; 1.0 mM EDTA; 0.05 mM DCPIP; 0.1 mM iP; 100 µg/ml BSA; and the sample tested. After the addition of the enzyme, absorbance change is read at 590 nm for 10 minutes.

After purification on the DEAE column, the major pooled active material was dialyzed against Buffer C (20 mM Tris, 0.5 M NaCl, 1.0 mM $CaCl_2$, pH 7.4), and applied to a concanavalin A agarose column (100 ml, from Sigma Aldrich, St. Louis, Missouri) and washed w/ Buffer C (270 ml). Glycosylated proteins were eluted with a step gradient of buffer C containing α-D-methylmannoside to 1 M over 400 ml. The relatively long retention time when eluted under these conditions indicates that the glycosylated form of *ckx1* was isolated. Active fractions from the lectin-affinity chromatography were then dialyzed against Buffer D (10 mM Tris, 1 mM EDTA, pH 8.5) and applied to a high resolution anion exchange column (FPLC MonoQ, 1 ml, from Amersham Pharmacia Biotech, Ltd., San Francisco, California), and eluted with a linear gradient of KCl to 0.12 M over 24 minutes at 1 ml/minute. The active fractions from the ion exchange column were then concentrated, dialyzed against Buffer B, brought to 1.5 M ammonium sulphate and applied to a hydrophobic interaction column (FPLC phenylsuperose, 1 ml, Pharmacia) equilibrated in Buffer B containing 0.6 ammonium sulfate. After washing with 0.6 M ammonium sulfate for 25 minutes, the concentration of ammonium sulfate was reduced successively to 0.45 M over 15 minutes and then to zero over 60 minutes.

Native gel electrophoresis was performed as illustrated in Ornstein (1964) and Davis (1964). Gels were then stained for cytokinin oxidase activity by the DCPIP procedure described above. Enzyme activity was revealed as a transparent band against a blue background. Denaturing SDS polyacrylamide gel electrophoresis was then carried out as illustrated in Laemmli (1970). When testing fractions for homogeneity, the gel was stained as described by Møller (1995). At the final purification step, the enzyme was stained with Coomassie Blue R250. The purified protein was analyzed by tryptic digestion, HPLC separation of digest, and Edman degradation

sequencing of the tryptic polypeptides. Several polypeptide sequences were obtained from this analysis, including SEQ. ID NOS. 4 and 5. From these sequences, reverse translation primer probes, SEQ. ID NOS. 6,7,8, and 9, were devised, with inosine substituted at highly degenerative positions. Primers SEQ. ID NOS. 6 and 9 were then combined with maize genomic DNA, and hot-start touchdown PCR was performed (Ault, 1994) for 40 cycles. PCR products were separated on agarose gel, and an approximately 440 bp fragment was chosen to use as a hybridization probe. The identity of the fragment was confirmed by showing that it could be amplified by PCR with the nested internal primers SEQ. ID NOS. 7 and 8. The fragment amplified by the primers SEQ. ID NOS. 6 and 9 was then ligated into linearized pCRII DNA and transformed into *E. coli* (INVαF', Invitrogen Corp., Carlsbad, California). After cloning and reisolation of DNA, plasmid inserts were sequenced using the Prism dideoxy terminator method of Applied Biosystems, Foster City, CA, to verify that the sequenced tryptic digest polypeptides were encoded by the fragment.

Once the large fragment had been verified, it was labeled with ^{32}P by primer extension using the Klenow fragment of DNA polymerase and primers SEQ. ID NOS. 6 and 9. Maize genomic library phage (in λ-FIXII, from Stratagene, La Jolla, California) were diluted in SM Buffer, and appropriate numbers added to freshly prepared E. coli (XL1-Blue MRA (P2), Stratagene) in 10 mM $MgSO_4$ and incubated at 37°C for 15 minutes. NZY top agar at 48°C was added and the mixture plated onto NZY agar plates. After incubation for approximately 8 hours at 37°C, plates were cooled to 4°C for 2 hours and phage were adsorbed onto sheets of Hybond N membrane (Amersham Pharmacia Biotech, San Francisco, California). Membranes were air-dried for 10 minutes, and incubated successively with 0.5 M NaOH plus 1.5 M NaCl, 500 ml, 5 minutes; 0.5 Tris-Cl pH 8.0 plus 1.5 M NaCl, 500 ml, 5 minutes; and 2 x SSC, 500 ml,

5 minutes. They were blotted dry and baked at 80°C until dry (approximately 15 minutes). Membranes were pre-hybridized at 45°C for less than one hour in 50% formamide, 5 x SSPE, 2 x Denhardt's solution, 0.2% SDS, 100-200 μg/ml denatured herring sperm or calf thymus DNA (Maniatis, 1990).

The labeled DNA fragment was then denatured at 100°C for 5 minutes, added to the pre-hybridization solution, and hybridized for 16 hours at 45°C. For the primary screen, phage were plated at a density of 500 pfu/cm^2 and membranes were washed at high stringency. For subsequent plaque purification, candidate phage were plated at a density of 70 pfu/cm^2 and 2 pfu/cm^2 and membranes were washed at medium stringency. After three rounds of purification, subfragments of the insert were removed from positive phage DNA by restriction and subcloned into pBluescript (Stratagene) for characterization by restriction digestion and sequence analysis. Two overlapping plasmid subclones, pROM2 (a HindIII insert) and pROM3 (an Xho-BamHI insert) each contained part of the gene for *ckx1*. These overlapping sections have been fused into the plasmid clone pROM10. The plasmids pROM2, pROM3, and pROM10 have been deposited with the American Type Culture Collection as ATCC Nos. 209573, 209572, and 209571, respectively. The sequence of the cloned DNA in pROM2 and pROM3 provided the genomic sequence of *ckx1*, SEQ. ID NO. 2, which was verified by the inclusion of sequences coding for the tryptic digest fragments obtained above.

The location of the introns in the *ckx1* gene, and the coding sequence for *ckx1* (SEQ. ID NO. 3) was verified by use of the reverse transcriptase polymerase chain reaction (RT-PCR) according to the following procedure.

Total RNA(2-4 μg, DNAase I-treated) from kernels harvested five days after pollination (5 DAP) was primed for RT-PCR using oligonucleotides bracketing the intron

sites. Primers 2031f (*ckx1* genomic sequence 2031-2050) and XBH1 (reverse complement of *ckx1* genomic sequence 2553-2570) covered the first intron site. Primers 3160f (*ckx1* genomic sequence 3160-3179) and 3484r (reverse complement of *ckx1* genomic sequence 3465-3484) covered the second intron site. Reverse transcription was at 50°C for 50 min. with 200U Superscript II (Gibco-BRL) in 20 μL total volume with 25 mM Tris-Cl, pH 8.3, 37.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 5mM dithiothreitol, 40U RNAasin (Promega, Madison, Wisconsin). PCR was performed with 4-10% of the RT reaction product as template and 1U Taq polymerase in 1X PCR buffer (Roche Diagnostics, Basel, Switzerland), 200 μM dNTPs, and 0.5 μM each primer. Reaction conditions were: an initial denaturation at 95° C for four minutes followed by thirty-five cycles of 95° denaturation for one minute, 60° annealing for one minute, and 72° extension for one minute. A final four minute extension was carried out at 72°. PCR products were resolved on 1.5% agarose gels. PCR products were excised from gels and sequenced to determine splice site junctions.

RT-PCR of maize kernel RNA (5 days after pollination) with primers designed to span the first and second intron locations demonstrated PCR product sizes consistent with splicing out of these introns in the mature oxidase mRNA. The primers bracketing the first intron should give a 539 bp product if the intron is present and a 127 bp product if it has been spliced out. Likewise, the primers bracketing the second intron should give a 324 bp product if the second intron is present and a 232 bp product if it has been spliced out. As shown in

Figure 2, the PCR products were 127 bp and 232 bp respectively, indicating that the introns had indeed been spliced out. Sequence analysis of the fragments confirmed that splicing had occurred as predicted.

The identity of the *ckx1* protein and gene were further verified by the following immunological technique.

Polyclonal antibodies were raised in goats to the peptide produced by expression of a *ckx1* gene fragment in *E. coli*. This avoided the presentation of a glycosylated surface when raising antibodies to the gene product and the problems encountered by Burch, et al., when raising antibodies to naturally occurring *ckx1*. Goat antibodies from immunized and unimmunized animals were partially purified by sodium sulfate precipitation (Willams, et al., 1967). Affinity columns were prepared by coupling these purified antibodies (2 mg) to Aminolink Plus gels (1 mL, Pierce) at pH 10, following the manufacturer's protocol. Activity depletion assays were performed by adding 0.2 mL (27 μg) of a maize ConA-fractioned oxidase preparation plus 0.8 mL phosphate buffered saline (PBS) to each column. The columns were capped and incubated for one hour at room temperature. Eluate was collected and assayed for cytokinin oxidase activity.

Antibodies to *ckx1* recognize the major maize cytokinin oxidase enzyme. A column containing immobilized anti-*ckx1*-fragment antibody was able to deplete cytokinin oxidase activity from a ConA-fractionated maize extract. A control column of unimmunized goat antibodies was not able to do so.

Example 2: Expression of *ckx1* in *Pichia pastoris*

ckx1 protein may be produced in bulk by the following procedure for use in applications such as the cytokinin assay of Example 3, or application to plant materials.

Expression of *ckx1* in *Pichia pastoris* was carried out in four stages:

Step 1. Removal of the introns from *ckx1*

Step 2. Removal of the maize promoter and construction of the appropriate expression cassette using the intron-less construct

Step 3. Transformation of the final construct into *Pichia* and

Step 4. Expression of *ckx1* in *Pichia*

Removal of the right-most intron was accomplished by splicing by overlap extension (Horton *et al.*, 1989) and for the left-most intron by ligation of suitable restriction fragments. The resulting intronless cassette was inserted into the *Pichia* expression vector pPICZ-A to give the expression construct pROM24 (FIG. 9). This construct was introduced into the requisite *Pichia* host and grown in the presence of methanol to induce *ckx1* expression. Appropriate control *Pichia* lines (containing the vector alone or a recombinant expressing human serum albumin) were grown in parallel.

No cytokinin oxidase activity was observed in *Pichia* cell lysates containing *ckx1* or the controls at any time during the growth curve. However, the *Pichia* line harboring *ckx1* expressed and secreted high levels of cytokinin oxidase activity into the growth medium. No activity was observed in control supernatants. This provided further verification that *ckx1* does indeed encode a cytokinin oxidase.

Step 1. Intron removal: The second intron (SEQ. ID NO. 2 residues 3219-3312) was removed by splicing by overlap extension with selected *ckx1* restriction fragments as templates to limit artifactual priming. The following table lists the primers and templates used.

Intron	Left Primer	Right primer	Template DNA
amplification #1	TGGGAATTCCATGGGGAGA TGGTGACGTGCTC (SEQ. ID NO. 11)	GCCGTCCCACATGGATTTGT TGAGGGGGTAGAC (SEQ. ID NO. 12)	pROM2 Nco1/PinA1 (700 bp) fragment
amplification #2	CTCAACAAATCCATGTGGG ACGACGGCATGTCGGCGG (SEQ. ID NO. 13)	GCGGTCTAGATCTAACTAAA ACATGCATGGGCTATCATC (SEQ. ID NO. 14)	pROM2 390 bp PinA1 + Vsp1
amplification #3	ATGGGAATTCCATGGGGAG ATGGTGACGTGCTC (SEQ. ID NO. 15)	GCGGTCTAGATCTAACTAAA ACATGCATGGGCTATCATC (SEQ. ID NO. 16)	Products from amplifications #1 and # 2

PCR products from reactions #1 and #2 were gel-purified and used in the final PCR step. The final product from reaction #3 was cloned and sequenced and a *Pf1M1/ Xba1* subfragment was substituted for the intron-containing *Pf1M1/ Xba1* subfragment of pROM7. The construct was designated pROM19. The first intron (SEQ. ID NO. 2 residues 2113-2524) was then removed from pROM19 by replacement of the DNA between the restriction sites *PinA1* and *Nco1* with a linker constructed from the oligonucleotides CCGGTTTTGGTACCGGT (SEQ. ID NO. 17) and CATGACCGGTACCAAAA (SEQ. ID NO. 18). The product was designated pROM20. Extraneous linker-associated bases were removed by digestion with PinA1 followed by re-ligation. The product, designated pROM22 (FIG. 8), contained the three fused *ckx1* exons and the maize *ckx1* promoter.

Step 2. The Pichia expression cassette: The maize promoter was removed by partial digestion of pROM22 (FIG. 8) with *AatII*, filling in the sticky ends with T4 DNA polymerase, and redigestion with *BglII*. The exon fusion

(containing *ckx1* and including its putative signal peptide) was ligated into the *Pml1/BsmB1* restriction sites of pPICZ-A (Easy-Select *Pichia* Expression Kit version B, Invitrogen Corp.). The resulting plasmid was designated pROM24 (FIG. 9).

Step 3. Transformation into Pichia strain X33 as described in the Invitrogen protocol: The plasmid pROM24 (FIG. 9) (10 μg) was digested with *Dra1* and electroporated into competent X33 cells in a 2 mM cuvette at 1.75 kV (GenePulser, Bio-Rad Laboratories, Hercules, California). Selection on YPDS with 100 μg/mL zeocin resulted in many colonies. One (PP*ckx1*) was selected for expression studies.

Step 4. Expression of the oxidase: The transformant PP*ckx1* was inoculated into BMGY medium (50 mL) and grown overnight. Cells were pelleted, resuspended in BMMY (containing 0.5% v/v methanol), diluted to an A_{600}=1 and grown at 30°C with vigorous shaking. Additional methanol was added to 0.5% v/v at 24, 48 and 72 hours post-inoculation. Samples were harvested for assay of cytokinin oxidase activity in cell lysates or in culture supernatants. *Pichia* strains X33 (WT, no insert) and GS115, (secreted human serum albumin insert) served as controls.

Example 3: Use of Recombinant Cytokinin Oxidase in a Rapid Assay Method for Cytokinin

Cytokinins were measured by mixing 100 μL of a buffer mixture containing phosphate buffer (250 mM, pH 7.0), EDTA (2.5 mM) and DCPIP (0.125 mM), and an excess of recombinant cytokinin oxidase with solutions (150 μL) of zeatin at various concentrations. The net change in absorbance was measured at 590 nm.

Figure 3 illustrates the change in absorbance when the assay is used to measure the cytokinin zeatin. The method is capable of measuring as little as 2 nmol zeatin but, the major advantage of the assay over the prior art is its rapidity. Assays can be preformed in as little as five minutes, significantly faster than radioimmunoassays (MacDonald and Morris, 1985). Further, the method can be integrated into cytokinin production systems by coupling the *ckx1* gene to such cytokinin producing genes as *ipt* or *tzs*, in order to assay cytokinin production *in vitro*.

Example 4: Unregulated and Regulated Expression of *ckx1* Constitutively and in the Roots of *Nicotiana tabacum*

The following procedure, which produces tobacco plants altered to express *ckx1* constitutively and in their roots, is a slight modification of the standard protocols described in Draper, et al., 1988.

Nicotiana tabacum cultivar Xanthi is a standard tobacco line. Disarmed *Agrobacterium tumefaciens* strains such as LBA4404 (Hoekema, et al., 1985) are used. Murashige and Skoog salts, phytagar, sucrose, etc. are reagent or tissue-culture grade.

Three separate constructs were made with the *ckx1* coding sequence (SEQ. ID NO. 3) to effect three different patterns of *ckx1* expression in transformed tobacco plants. Constructs were based on the BIN19 plasmid primary vector (which includes an *Agrobacterium* compatible replication origin and kanamycin selection markers, Bevan, et al., 1984). The following constructs were made:

CaMV-ckx1-nos: The cauliflower mosaic virus promoter with a *nos* enhancer (U.S. Patent No. 5,530,196), are present in pBI121 (FIG. 5)(available from Clonetech, Palo

Alto, California). The β-glucuronidase gene of pBI121 was excised with a *BamHI/EcoIcrI* digest. The coding sequence of *ckx1* (SEQ. ID NO. 3) was obtained by a *BglII* digest of pROM26 (FIG. 10)(pROM24 (FIG. 9) altered by site-directed mutagenesis to contain another *BglII* and an *XhoI* restriction site). The final construct pROM30 (FIG. 13), which induces strong constitutive expression of *ckx1*, was created by digesting the altered pBI121 with *BamHI* and ligating in the *ckx1* coding sequence.

CaMV-tet-ckx1-ocs: The *ckx1* coding region of pROM26 (FIG. 10) was isolated by *BglII* digestion and ligated into the *BamHI* site of pUCA7-TX (Gätz, et al., 1992) to form pROM32 (FIG. 14). The tetracycline-regulated operator element (U.S. Patent No. 5,464,758) from pUCA7-TX and the inserted coding region were excised from pROM32 (FIG. 14) by *PvuII* digestion. The final construct pROM29 (FIG. 12), was produced by ligating the excerpt into the *SalI* site of pJL7 (FIG. 4)(a BIN19 type plasmid). When this construct is introduced into tobacco previously transformed to express the *tet* repressor protein, no *ckx1* activity will be expressed until repression is relieved by addition of tetracycline. This construct may be of particular use in host organisms where strong constitutive expression of *ckx1* results in abnormal growth patterns.

RB7-ckx1-nos: The tobacco *RB7* root specific promotor (U.S. Patent No. 5,750,386), was obtained by the following method. A fragment containing the promoter was PCR-amplified from tobacco genomic DNA using GACACCATTCCAAGCATACCCC (SEQ. ID NO. 19) and GTTCTCACTAGAAAAATGCCCC (SEQ. ID NO. 20) as primers. This 1400 bp product was ligated into the pCRII plasmid

(Invitrogen) to produce pROM8 (FIG. 6). The *HindIII-EcoRI* region of the insert, containing the nematode-specific portion of the promoter, was then excised and ligated into the *HindIII-EcoRI* site of pBluescriptII KS+ (Stratagene) to produce pROM9 (FIG. 7). The *EcoRI-NsiI* fragment of pROM24 (FIG. 9), containing the *ckx1* coding sequence, was excised and ligated into the *EcoRI-PstI* site of pROM9, to make pROM28 (FIG. 11). The final construct pROM43 (FIG. 15), which induces expression of *ckx1* in roots when the transformed plants' roots are attacked by nematodes, was produced by ligating a *HindIII-SacI* fragment of pROM28 into the *HindIII-SacI* site of pBI121 (FIG. 5).

Media Component	MSS	PC	SI	MSSK
MS Salts (g/l)	4.3	4.3	4.3	4.3
Sucrose (g/l)	30.0	30.0	30.0	30.0
Phytagar (g/l)	7.5	5.0	5.0	7.5
NAA (mg/l)	0	1.0	1.0	0
BAP (mg/l)	0	0.1	0.1	0
Timetin (mg/l)	0	0	200	200
Kanamycin (mg/l)	0	0	50.0	50.0
pH	5.7	5.7	5.7	5.7

Nicotiana was continuously maintained by axenic shoot tip culture on MSS and sub-cultured at 4 week intervals.

The three binary plasmid constructs described above were electroporated into the disarmed *A. tumefaciens* host and transformants were grown under suitable selection (An, et al. 1985). Late-log phase cultures were used for transformation.

Young axenic tobacco leaves (3 to 4 weeks after tip culture) were dissected into 4 to 6 segments, excluding

the largest vasculature. Segments were cultured on PC, abaxial side up, for 48 hours. The leaf pieces were then soaked in transformed *A. tumefaciens* culture diluted with water (1:1), for 1 hour. Pieces were then placed on the original PC medium for 48 hours. The leaves were then washed with water thoroughly and placed on SI medium and removed to fresh SI medium every 7 to 10 days. Although no adverse developmental effects were observed with *Nicotiana* transformants, the use of a non-substrate cytokinin such as benzylaminopurine, or tetracycline repression, may be needed for the culture of some host plants. Shoots were removed onto MSSK media when 1 cm long. Successive tip culture was carried out for 2 to 3 transfers, after which the transgenic plants were maintained on MSS media.

In light of the detailed description of the invention and the examples presented above, it can be appreciated that the several objects of the invention are achieved. The explanations and illustrations presented herein are intended to acquaint others skilled in the art with the invention, its principles, and its practical application. Those skilled in the art may adapt and apply the invention in its numerous forms, as may be best suited to the requirements of a particular use. Accordingly, the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention.

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WE CLAIM AS OUR INVENTION:

1. A substantially purified protein which exhibits cytokinin oxidizing activity, selected from the group consisting of:

(a) SEQ. ID NO. 1,

(b) a protein having an amino acid sequence which includes the amino acid sequence of SEQ. ID NO. 1,

(c) a protein having an amino acid sequence which includes a portion of the amino acid sequence of SEQ. ID NO. 1, the included portion being at least about 20 amino acid residues in length and conferring the cytokinin oxidizing activity on the protein, and

(d) a protein including an amino acid sequence with at least about 65% sequence identity to SEQ. ID NO. 1, the remainder of amino acid residues being conservatively substituted.

2. The protein of claim 1 wherein the protein is purified.

3. The protein of claim 1 which is SEQ. ID NO. 1.

4. The protein of claim 1 produced by a host cell transformed with a vector containing a nucleic acid polymer which encodes the protein of claim 1.

5. A substantially isolated nucleic acid polymer encoding a protein of claim 1.

6. The substantially isolated nucleic acid polymer of claim 5 which encodes SEQ. ID NO. 1.

7. A substantially isolated nucleic acid polymer which encodes a protein which exhibits cytokinin oxidizing activity, wherein the nucleic acid polymer is selected from the group consisting of:

(a) SEQ. ID NO. 2,

(b) SEQ. ID NO. 3,

(c) a nucleic acid polymer with a sequence described by SEQ. ID NO. 10,

(d) a nucleic acid polymer whose sequence contains a sequence selected from the group consisting of SEQ. ID NO. 2, SEQ. ID NO. 3, or a nucleic acid polymer with a sequence described by SEQ. ID NO. 10.

(e) a nucleic acid polymer which contains at least one 60 base pair portion, which encodes an amino acid sequence which confers cytokinin oxidizing activity upon the encoded protein, of a nucleotide sequence selected from the group consisting of SEQ. ID NO. 2, SEQ. ID NO. 3, and a nucleic acid polymer with a sequence described by SEQ. ID NO. 10,

(f) a nucleic acid polymer which encodes a protein including an amino acid sequence with at least about 65% sequence identity to SEQ. ID NO. 1, the remainder of amino acid residues being conservatively substituted,

(g) a nucleic acid polymer which hybridizes to a nucleotide sequence selected from the group consisting of SEQ. ID NO. 2, SEQ. ID NO. 3, and a nucleic acid polymer with a sequence described by SEQ. ID NO. 10, under a wash stringency equivalent to 0.5X to 2X SSC buffer, 0.1% SDS, at 55-65°C, and

(h) a nucleic acid polymer having a nucleotide sequence complementary to any of the nucleic acid sequences (a) - (g).

8. The substantially isolated nucleic acid polymer of claim 7 wherein the polymer contains a sequence described by SEQ. ID NO. 10 or a sequence complementary to a sequence described by SEQ. ID NO. 10.

9. The substantially isolated nucleic acid polymer of claim 7 wherein the polymer is SEQ. ID No. 2 or its complementary sequence.

10. The substantially isolated nucleic acid polymer of claim 7 wherein the polymer is SEQ. ID No. 3 or its complementary sequence.

11. A host cell transformed with a vector containing a deoxyribonucleic acid polymer which encodes the protein of claim 1.

12. The host cell of claim 11, wherein the host cell is a plant cell.

13. The host cell of claim 12, wherein the host plant cell is selected from the group consisting of:

(a) *Nicotiana tabacum*,

(b) *Arabidopsis thaliana*,

(c) *Zea mays*

(d) *Brassica spp*

(e) *Oryza sativa*

14. The host cell of claim 11 wherein the host cell is selected from the group consisting of:

(a) *Pichia pastoris*

(b) *Escherichia coli*

15. A plant regenerated from the host plant cell in claim 12.

16. A plant product from the regenerated plant of claim 15.

17. The plant product of claim 16, wherein said plant product is selected from the group consisting of seeds, leaves, stem cultures, rhizomes, and bulbs.

18. A method for producing the protein of claim 1 in a host cell, essentially comprising

(a) constructing a vector containing DNA encoding the protein of claim 1 and an expression regulatory sequence operational in the host cell;

(b) transfecting a host cell suitable for protein production with the vector; and

(c) expressing the protein in the host cell.

19. The method of claim 18 wherein the vector is selected from the group consisting of:

(a) a plasmid comprised of DNA,

(b) *Agrobacterium tumefaciens*

20. The method of claim 18, wherein the host cell is selected from the group consisting of:

(a) *Pichia pastoris*

(b) *Escherichia coli*

21. The method of claim 18 wherein the host cell secretes said protein into a culture medium, and wherein the method further comprises purifying said protein from the culture medium.

22. The method of claim 18 further comprising the step of reconstituting the host cell into an organism.

23. The method of claim 18, wherein the host cell is a plant cell.

24. The method of claim 23, wherein the host plant cell is selected from the group consisting of:

(a) *Nicotiana tabacum*,

(b) *Arabidopsis thaliana*,

(c) *Zea mays*

(d) *Brassica spp*

(e) *Oryza sativa*

25. A method of moderating cytokinin-associated pathogenesis in a plant comprising transforming a plant cell with a nucleic acid polymer construct which contains:

(a) a nucleic acid polymer encoding the protein of claim 1, and

(b) an expression regulatory sequence operational in the host plant; and

regenerating a plant from the transformed plant cell.

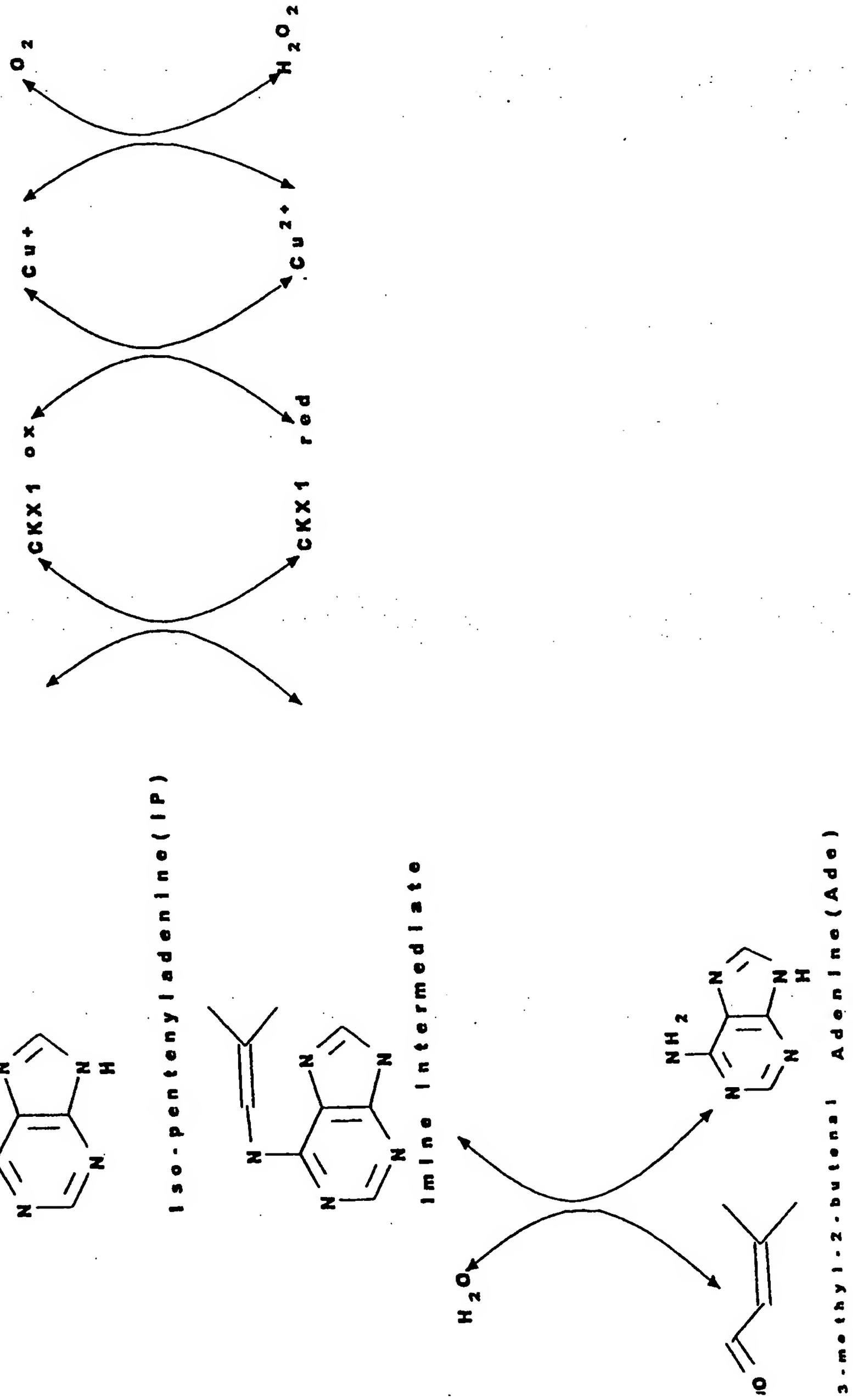
26. A method as set forth in claim 25 wherein the expression regulatory sequence is selected from the group consisting of constitutive promoters, inducible promoters, repressors, and enhancers.

27. A method of detecting cytokinin concentrations in a sample essentially comprising:

(a) adding to a buffered sample known amounts of dichlorophenolindophenol and an excess IU activity producing amount of the protein of claim 1;

(b) measuring the net change in absorbance of light at 590 nm; and

(c) comparing this net absorbance to a standard curve generated from known concentrations of cytokinin.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Cu+
Cu2+
CKX1 ox
CKX1 red
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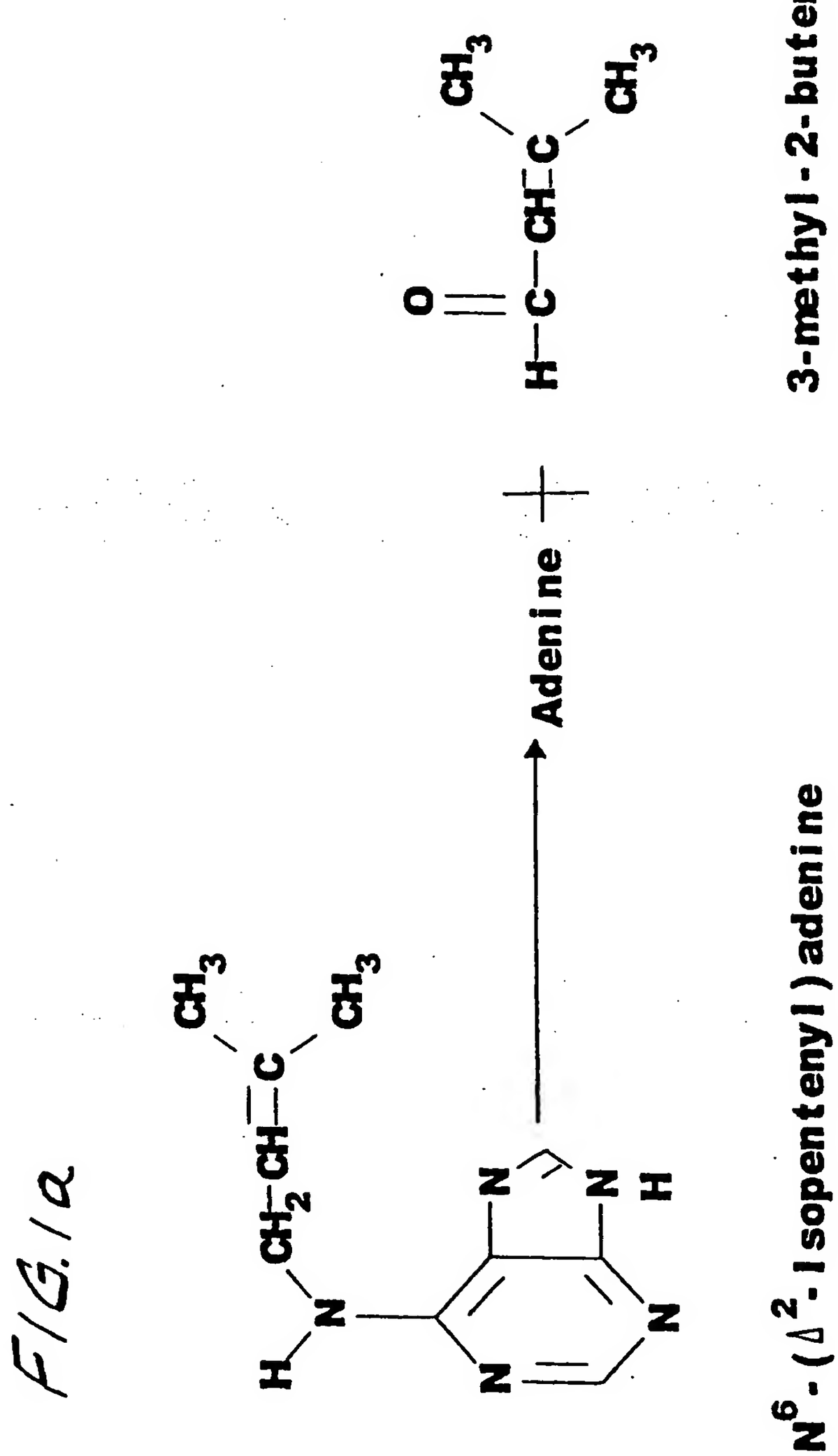
Adenine(Ado)
NH2
H2O
O

FIG. 1b

FIG. 1a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O
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N
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FIG. 2A
VERIFICATION OF ckx1 GENE INTRON LOCATIONS BY RT-PCR

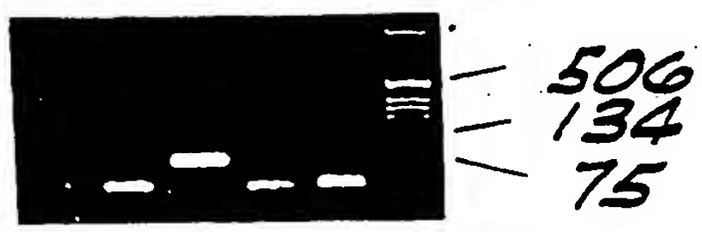


FIG. 2B
VERIFICATION OF ckx1 GENE INTRON LOCATIONS BY RT-PCR

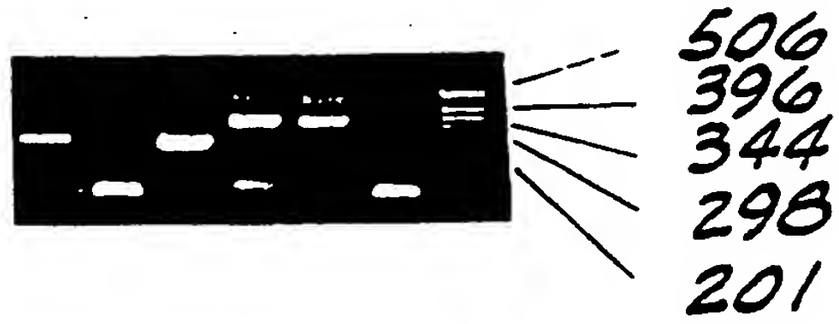
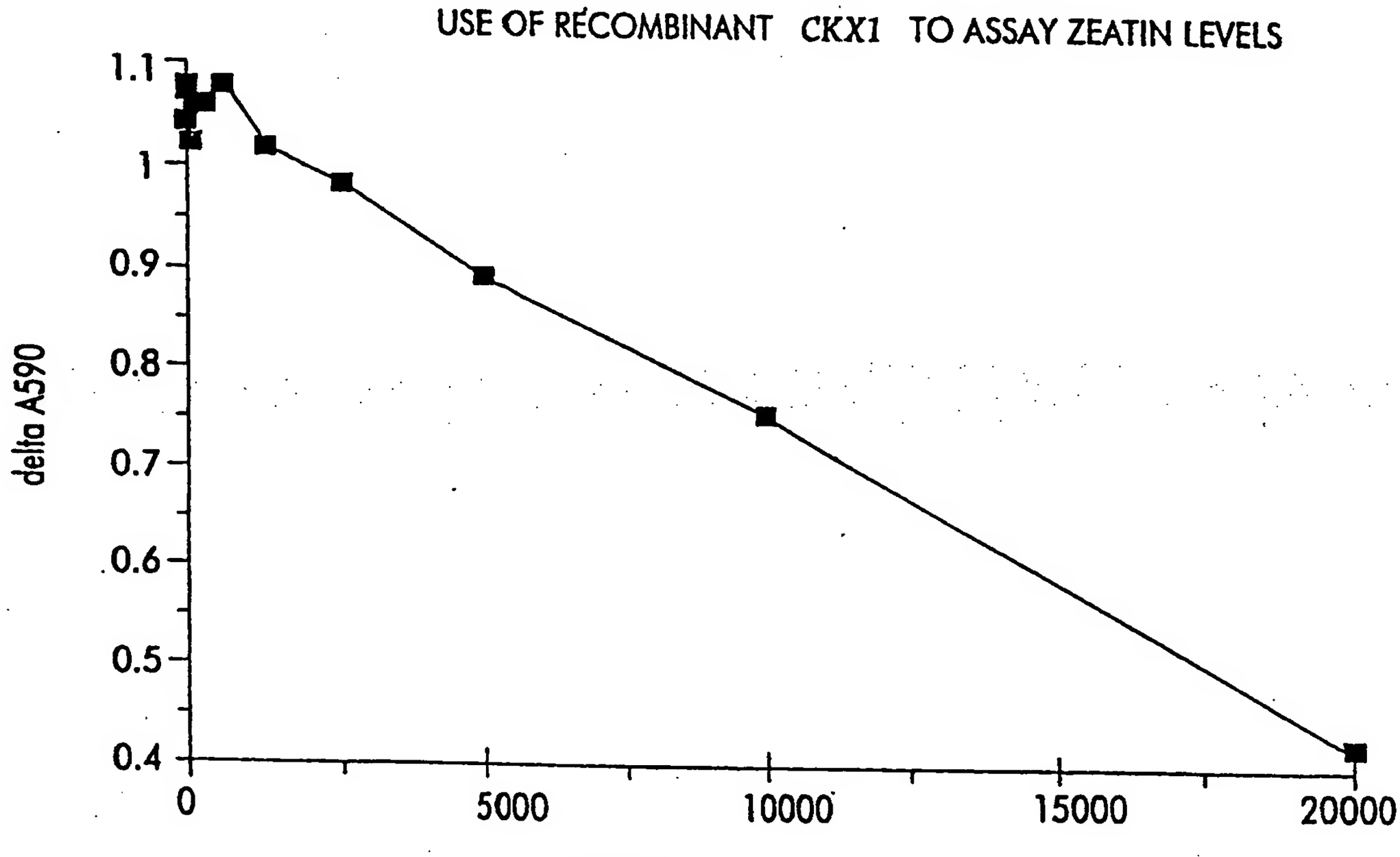


FIG.3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0.4
0
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10000
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ZEATIN (pmol)

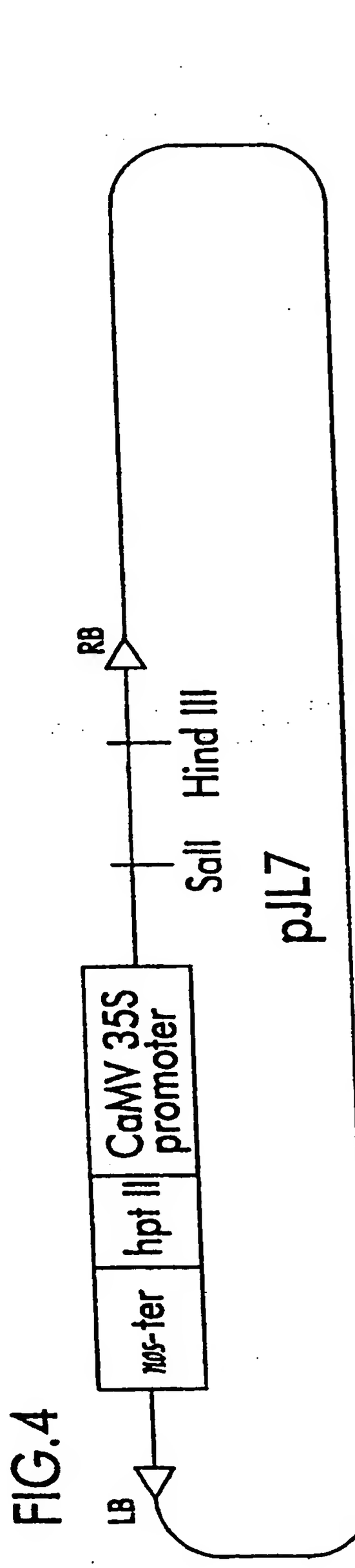


FIG.4

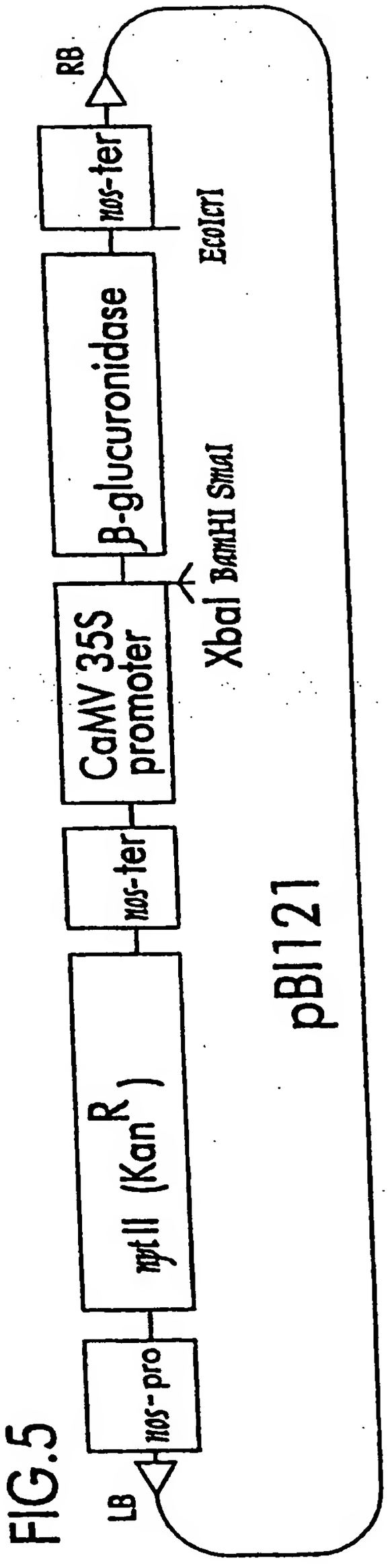


FIG.5

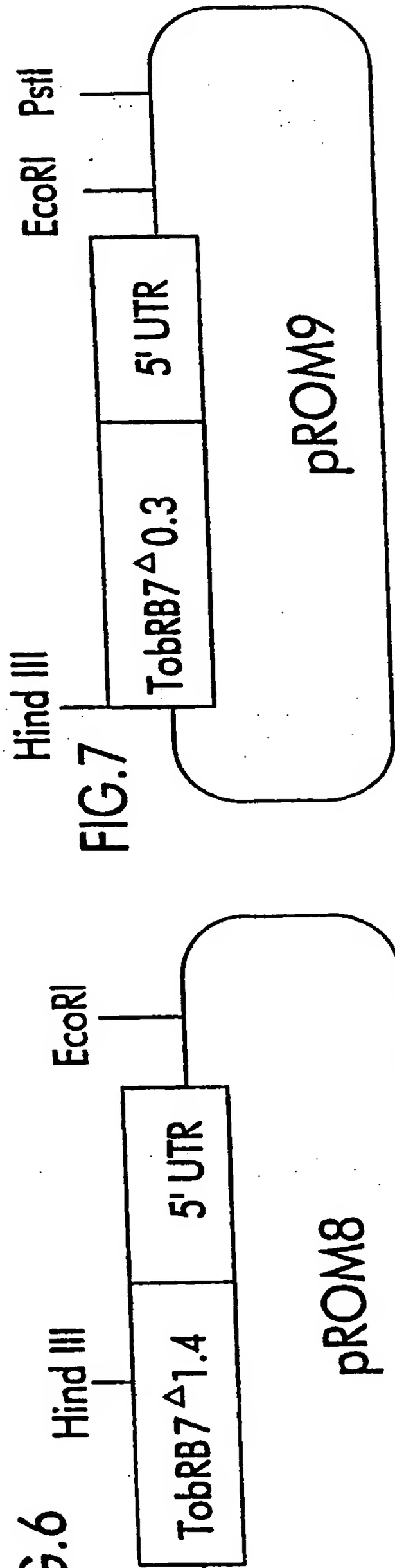


FIG.7

FIG.6

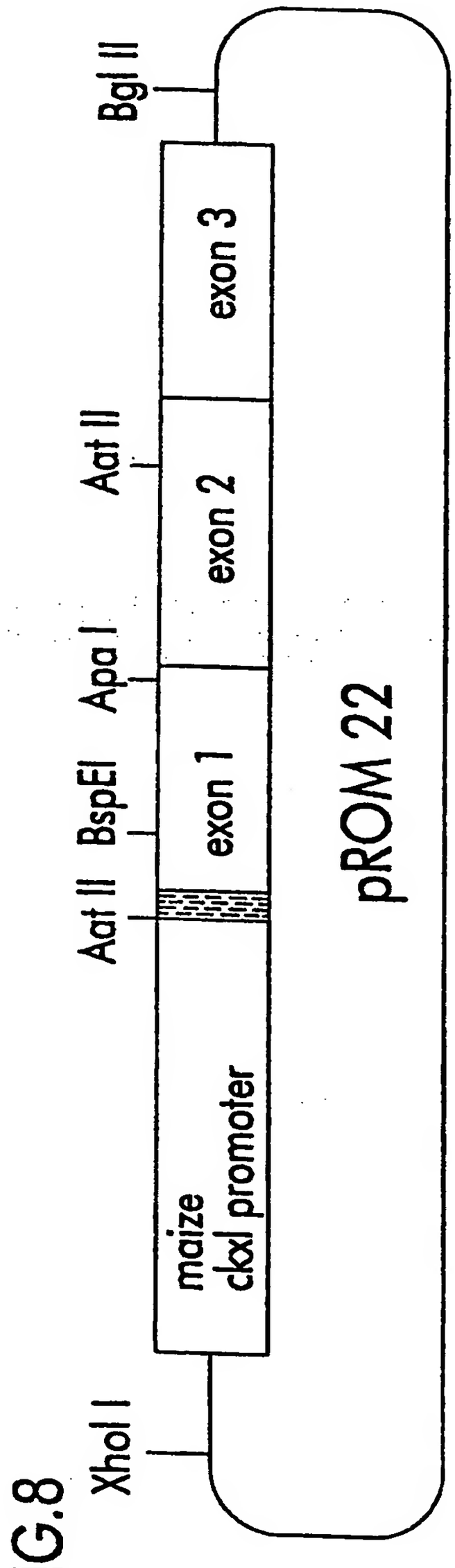


FIG.8

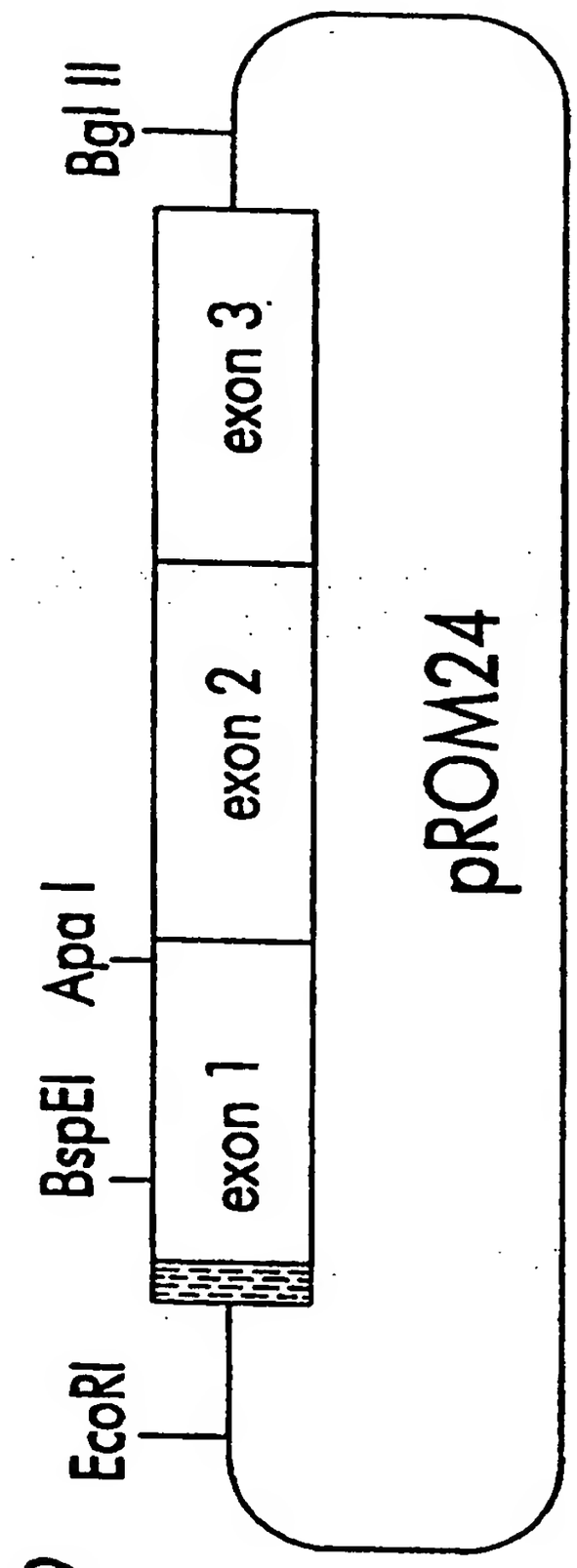


FIG.9

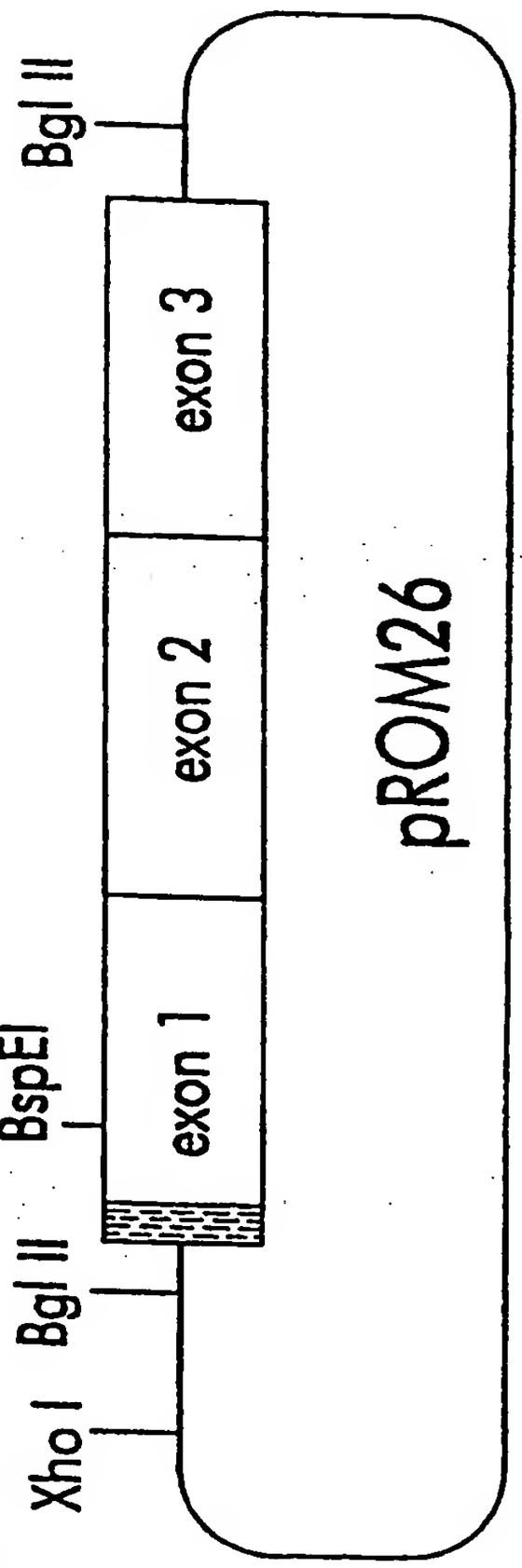


FIG.10

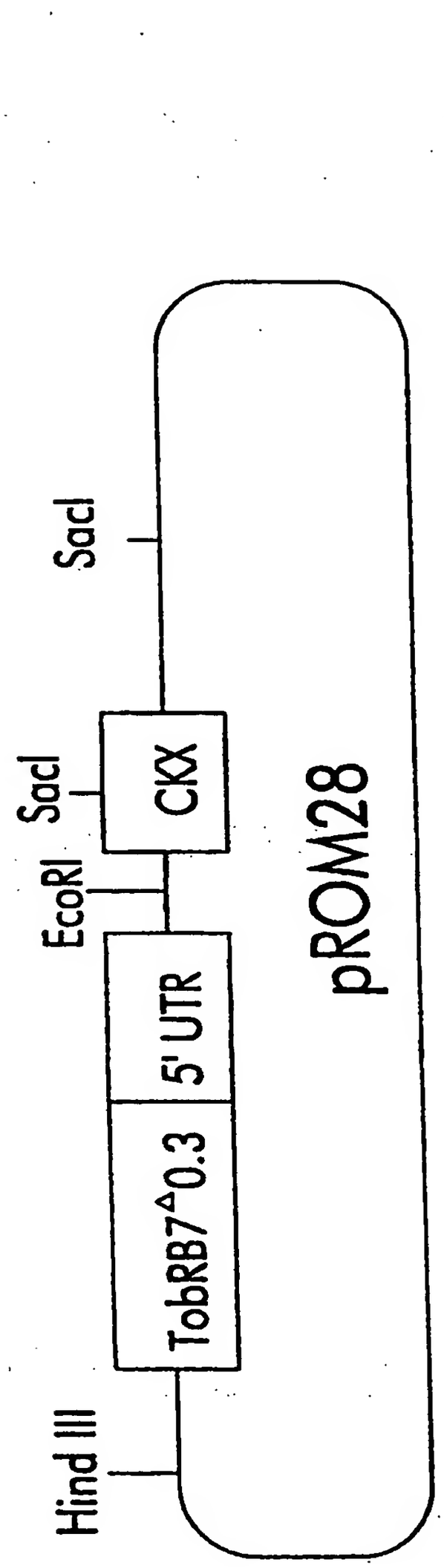


FIG.11

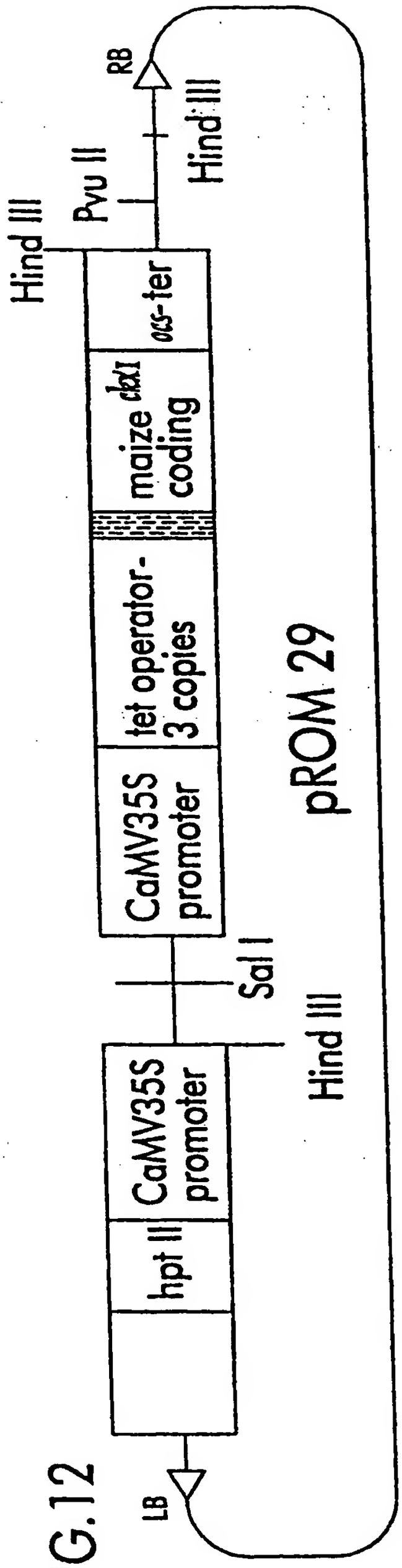


FIG.12

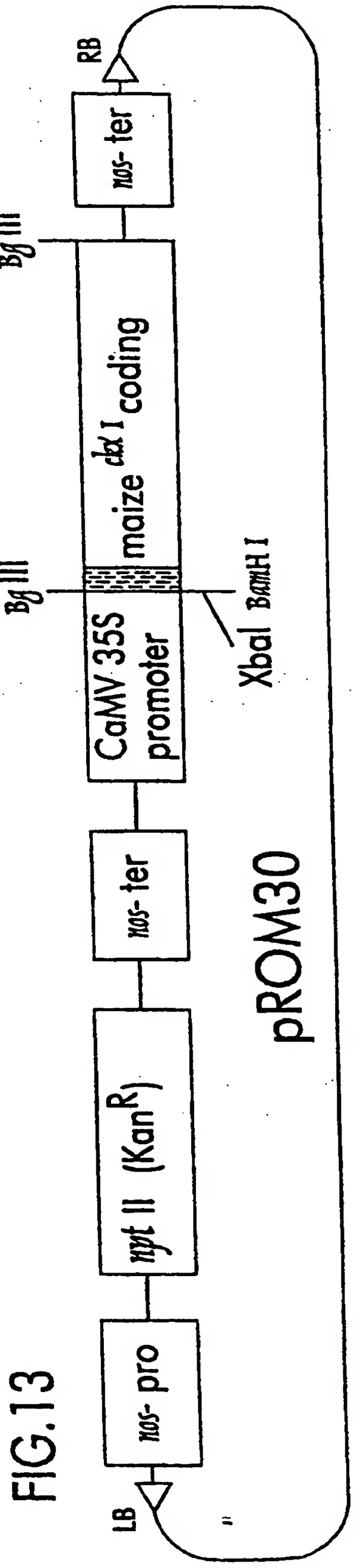
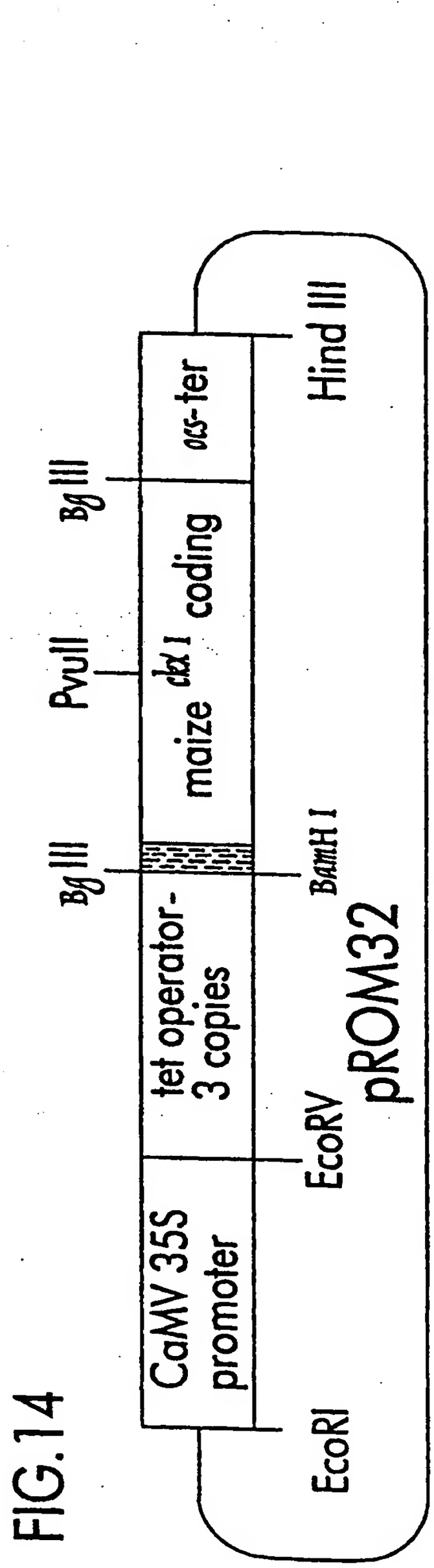


FIG.13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CaMV 35S promoter
EcoRV
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Bg III
maize dx1 coding
Pvull
Bg III
ocs-ter
Hind III

FIG.14

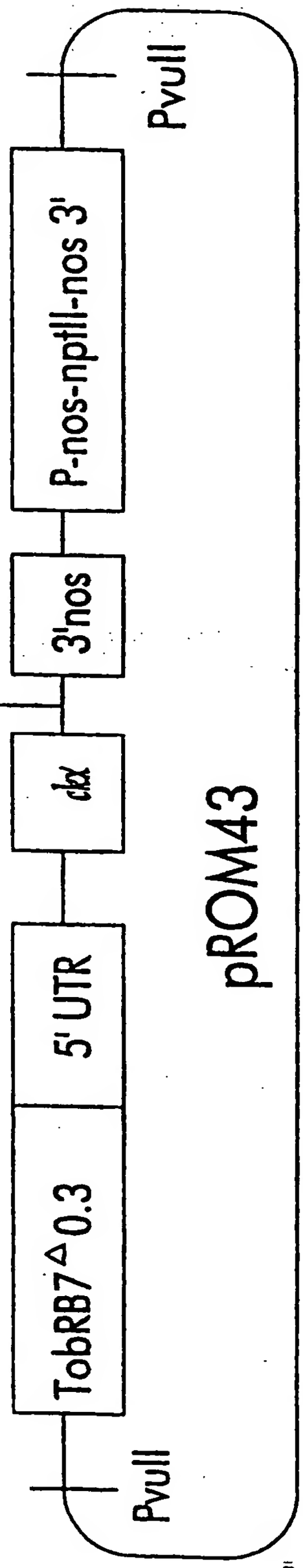
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FIG.15

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Asn Lys Ser
    435

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His Ala Leu Ala Ala Gly Thr Pro Ala Leu Gly Asp Asp Arg Gly Arg
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ccc tgg cca gcc tcc ctc gcc gcg ctg gcc ttg gac ggc aag ctc cgg   144
Pro Trp Pro Ala Ser Leu Ala Ala Leu Ala Leu Asp Gly Lys Leu Arg
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acc gac agc aac gcg acg gcg gcg gcc tcg acg gac ttc ggc aac atc   192
Thr Asp Ser Asn Ala Thr Ala Ala Ala Ser Thr Asp Phe Gly Asn Ile
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acg tcg gcg ctc ccg gcg gcg gtc ctg tac ccg tcg tcc acg ggc gac   240
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ctg gtg gcg ctg ctg agc gcg gcc aac tcc acc ccg ggg tgg ccc tac   288
Leu Val Ala Leu Leu Ser Ala Ala Asn Ser Thr Pro Gly Trp Pro Tyr
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acc atc gcg ttc cgc ggc cgc ggc cac tcc ctc atg ggc cag gcc ttc   336
Thr Ile Ala Phe Arg Gly Arg Gly His Ser Leu Met Gly Gln Ala Phe
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gcc ccc ggc ggc gtc gtc gtc aac atg gcg tcc ctg ggc gac gcc gcc   384
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Gly Val Ala Pro Arg Ser Trp Asn Asp Tyr Leu Tyr Leu Thr Val Gly
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ggc acg ctg tcc aac gca ggc atc agc ggc cag gcg ttc cgc cac ggc   576
Gly Thr Leu Ser Asn Ala Gly Ile Ser Gly Gln Ala Phe Arg His Gly
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cca cag ata tct aac gtg ctg gag atg gac gtt atc acc ggc cat ggg   624
Pro Gln Ile Ser Asn Val Leu Glu Met Asp Val Ile Thr Gly His Gly
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gag atg gtg acg tgc tcc aag cag ctg aac gcg gac ctg ttc gac gcc   672
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gcg gtg gag ccg gcg ccg gcg cgg gcg cgg tgg gtg cgg ttc gtg tac   768
Ala Val Glu Pro Ala Pro Ala Arg Ala Arg Trp Val Arg Phe Val Tyr
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acc gac ttc gcg gcg ttc agc gcc gac cag gag cgg ctg acc gcc ccg   816
Thr Asp Phe Ala Ala Phe Ser Ala Asp Gln Glu Arg Leu Thr Ala Pro
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Gly Ser Val Phe Val Asn Gln Ser Leu Ala Thr Asp Leu Ala Asn Thr
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Gly Phe Phe Thr Asp Ala Asp Val Ala Arg Ile Val Ala Leu Ala Gly
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gag cgg aac gcc acc acc gtg tac agc atc gag gcc acg ctc aac tac  1008
Glu Arg Asn Ala Thr Thr Val Tyr Ser Ile Glu Ala Thr Leu Asn Tyr
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gac aac gcc acg gcg gcg gcg gcg gcg gtg gac cag gag ctc gcg tcc  1056
Asp Asn Ala Thr Ala Ala Ala Ala Ala Val Asp Gln Glu Leu Ala Ser
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Val Leu Gly Thr Leu Ser Tyr Val Glu Gly Phe Ala Phe Gln Arg Asp
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Val Ala Tyr Ala Ala Phe Leu Asp Arg Val His Gly Glu Glu Val Ala
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ctc aac aag ctg ggg ctg tgg cgg gtg ccg cac ccg tgg ctc aac atg  1200
Leu Asn Lys Leu Gly Leu Trp Arg Val Pro His Pro Trp Leu Asn Met
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ggc atc ctg cag ggc acc gac atc gtc ggc ccg ctc atc gtc tac ccc   1296
Gly Ile Leu Gln Gly Thr Asp Ile Val Gly Pro Leu Ile Val Tyr Pro
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ctc aac aaa tcc atg tgg gac gac ggc atg tcg gcg gcg acg ccg tct   1344
Leu Asn Lys Ser Met Trp Asp Asp Gly Met Ser Ala Ala Thr Pro Ser
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Glu Asp Val Phe Tyr Ala Val Ser Leu Leu Phe Ser Ser Val Ala Pro
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Cys Asp Leu Ala Gly Ile Gln Tyr Lys Thr Tyr Leu Ala Arg His Thr
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Asp Arg Ser Asp Trp Val Arg His Phe Gly Ala Ala Lys Trp Asn Arg
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INTERNATIONAL SEARCH REPORT

Intr tional Application No
PCT/US 98/15844

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N15/81 C12N9/06 C12N1/21
C12N1/19 C12N5/10 A01H5/00 C12Q1/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 119, no. 21, 22 November 1993 Columbus, Ohio, US; abstract no. 221752, BURCH, L. R. ET AL: "Cytokinin oxidase and the degradative metabolism of cytokinins" XP002086612 see abstract & PHYSIOL. BIOCHEM. CYTOKININS PLANTS, SYMP. (1992), MEETING DATE 1990, 29-32. EDITOR(S): KAMINEK, MIROSLAV;MOK, DAVID W. S.; ZAZIMALOVA, EVA. PUBLISHER: SPB ACAD. PUBL., THE HAGUE, NETH. CODEN: 59KXA9, --- -/--	1-4

[X] Further documents are listed in the continuation of box C.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 December 1998	18. 01. 99
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer MADDOX, A

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INTERNATIONAL SEARCH REPORT

In' ational Application No
PCT/US 98/15844

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHREIBER B M N ET AL: "Polyclonal antibodies to maize seedling cytokinin oxidase" ANNUAL MEETING OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, CHARLOTTE, NORTH CAROLINA, USA, JULY 29-AUGUST 2, 1995. PLANT PHYSIOLOGY (ROCKVILLE) 108 (2 SUPPL.). 1995. 80., XP002086604	1-4
A	see the whole document	5-24

X	BURCH, LINDSAY R. ET AL: "The purification of cytokinin oxidase from Zea mays kernals" PHYTOCHEMISTRY (1989), 28(5), 1313-19, XP002086605	1-4
Y	see the whole document	5-24

Y	MEILAN R ET AL: "Cloning the cytokinin oxidase gene." ANNUAL MEETING OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, PORTLAND, OREGON, USA, JULY 30-AUGUST 3, 1994. PLANT PHYSIOLOGY (ROCKVILLE) 105 (1 SUPPL.). 1994. 68., XP002086606	5-24
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	see page 162	

P,X	MORRIS, R.O, ET AL.: "Zea mays cytokinin oxidase (ckx1) gene, complete cds" EMBL SEQUENCE ACCESSION NO. AF044603, 27 July 1998, XP002086607	1-11
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Annex to Form PCT/ISA/206
COMMUNICATION RELATING TO THE RESULTS OF THE PARTIAL INTERNATIONAL SEARCH

International Application No
PCT/EP 01/06833

1. The present communication is an Annex to the invitation to pay additional fees (Form PCT/ISA/206). It shows the results of the international search established on the parts of the international application which relate to the invention first mentioned in claims Nos.:
see 'Invitation to pay additional fees'
2. This communication is not the international search report which will be established according to Article 18 and Rule 43.
3. If the applicant does not pay any additional search fees, the information appearing in this communication will be considered as the result of the international search and will be included as such in the international search report.
4. If the applicant pays additional fees, the international search report will contain both the information appearing in this communication and the results of the international search on other parts of the international application for which such fees will have been paid.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 06571 A (UNIV MISSOURI) 11 February 1999 (1999-02-11) cited in the application	1,2
Y	the whole document	7-10, 13-32, 34,35, 39-62

X	ROY O. MORRIS ET AL.,: "Isolation of a gene encoding a glycosylated cytokinin oxidase from maize" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 255, 1999, pages 328-333, XP002151605 cited in the application	3-6,11, 12
Y	* the whole document, in particular figure 6 *	7-10, 13-32, 34,35, 39-62

X	DATABASE SWALL 'Online! 1 January 1998 (1998-01-01) S.D. ROUNSLEY ET AL.,: XP002151606 Database Accession number ID/AC=022213 abstract	3-6,11, 12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Annex to Form PCT/ISA/206
COMMUNICATION RELATING TO THE RESULTS OF THE PARTIAL INTERNATIONAL SEARCH

International Application No
PCT/EP 01/06833

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE SWALL 'Online! 1 May 2000 (2000-05-01) M. BEVAN ET AL.,: "Cytokinin oxidase-like protein" XP002151607 Database Accession number ID/AC=Q9SU77 abstract ---	3-6,11, 12
X	DATABASE SWALL 'Online! 1 May 1999 (1999-05-01) X. LIN ET AL: "Arabidopsis thaliana chromosome II BAC F3P11 genomic sequence. Putative Cytokinin Oxidase" XP002151608 Database Accession number ID/AC=Q9ZUP1 abstract ---	3-6,11, 12
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; M. FRANK ET AL.,: "TSD genes negatively regulate merismetic activity in Arabidopsis" XP002151616 Database Accession number AN=PREV200000242628 * abstract * & Biologia Plantarum (Prague) 1999, Vol. 42 (Suppl.), page S47 ---	11,12
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; ZHANG, N. ET AL.,: "Initiation and elongation of lateral roots in Lactuca sativa" XP002151609 Database Accession number AN=PREV199900326622 * abstract * &International J. of Plant Sciences 1999, Vol. 160(3), pages 511-519 ---	1,2, 15-30, 39-62

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Form PCT/ISA/206 (Annex, continuation sheet)(July 1992)

Annex to Form PCT/ISA/206
COMMUNICATION RELATING TO THE RESULTS
OF THE PARTIAL INTERNATIONAL SEARCH

International Application No
PCT/EP 01/06833

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; Y. KODA ET AL.,: "Cytokinin production by tomato root. Identification of a major cytokinin produced by the root and environmental fcators affecting the production" XP002151610 Database Accession number AN=PREV198988038194 * abstract * & J. of the Faculty of Agriculture Hokkaido University 1989, Vol. 64(1), pages 10-20 -----	1,2, 15-30, 39-62

Form PCT/ISA/206 (Annex, continuation sheet)(July 1992)

Patent Family Annex

Information on patent family members

International Application No
PCT/EP 01/06833

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9906571 A	11-02-1999	US 6229066 B1	08-05-2001
		AU 8603898 A	22-02-1999
		BR 9811592 A	19-09-2000
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